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TITLE: Restoration of Immune Surveillance in Lung Cancer by Natural Killer Cells

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Julie Y. Djeu

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14. ABSTRACT

The goal of this application is to investigate how a microRNA, namely miR183, can disrupt the expression of a critical molecule, DAP12, that controls tumoricidal function in human Natural Killer (NK) Cells and to understand how nicotine, contained in tobacco smoke, utilizes this mechanism to abort immunity against lung cancer. In addition, we seek to explore the viability of targeting miR183 to restore NK cells as a new form of immunotherapy for early stage lung cancer. We have encountered problems with nicotine instability and work is still in progress to identify conditions to probe the effect of nicotine on NK cells. However, nicotine instability is not an issue in vivo and we have collected blood samples from tobacco smokers, e-cigarette users and past smokers to compare with never smokers for NK function. In terms of anti-miR183 therapeutics, we found that PLGA nanoparticles are readily taken up by NK cells but do not enter the lysosome, thus are not degraded within the NK cell. Nanoparticles will next be loaded with anti-sense miR183 to confirm that NK cell preloaded with anti-miR183 will resist TGFb suppression.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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1. **INTRODUCTION:**

Immune escape has emerged as one of the hallmarks of cancer and conquering this barrier is critical to early resistance against cancer. However, its mechanism remains obscure, especially related to natural killer (NK) cells. The goal of this application is to uncover how a microRNA, namely miR183, can disrupt the expression of a critical molecule, DAP12, that controls tumoricidal function in human Natural Killer (NK) Cells and to understand how nicotine, contained in tobacco smoke, utilizes this mechanism to abort immunity against lung cancer. In addition, we seek to explore the viability of targeting miR183 to restore NK cells as a new form of immunotherapy for early stage lung cancer. The specific aims are 1) to decipher the role of miR183 in nicotine-directed inhibition of human NK cell function, 2) to examine if the use of tobacco or nicotine-containing e-cigarettes induces miR183 expression and disrupt NK function, 3) to determine if targeting miR183 can serve as an immunotherapeutic modality for treating early stage lung cancer. These aims will be achieved through (i) analysis of molecular basis for nicotine control of the miR183 promoter, (ii) analysis of heavy tobacco smokers and e-cigarette smokers as well as former heavy smokers for loss of NK function, and (iii) analysis of a human xenograft *Nod-scid-IL2Rg*$^{-/-}$ (NSG) mouse model for the ability of human NK cells to respond to NK-directed nanoparticles bearing antagomiRs against miR183 to provoke human lung tumor regression in vivo. Such approaches will yield new insight into the pathogenic role of miR183 in nicotine-derived NK cell suppression and define a new miR-based immunotherapeutic strategy to treat lung cancer.

KEYWORDS: Natural Killer Cell, NK receptors, tumor microenvironment, Transforming Growth Factor-beta, nicotine, tobacco smokers, e-cigarette-users, lung cancer, microRNA-183, DAP12, NKp44, NKp46, nanoparticle-based immunotherapy

2. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Lung cancer mortality remains a leading therapeutic issue because of the heterogeneity of cell types and the diversity of genetic changes involved. New drugs offer an improved overall survival to only a small subset of patients and the majority of lung cancer patients can only be treated with palliative chemotherapy. Immune surveillance, on the other hand, can be effective against any form of lung cancer, if mobilized in the early stages of disease. Natural killer (NK) cells are particularly effective as the first line of innate immunity against cancer. However, we recently made the seminal finding that NK cells in the lung tumor microenvironment are significantly at a disadvantage because of loss of DAP12, a critical adaptor protein that anchors activating NK receptors on the cell surface to recognize tumor cells. This loss is caused by transforming growth factor beta (TGFb) produced by tumor cells that can induce microRNA (miR)-183 in infiltrating NK cells to downregulate DAP12 expression. In addition to TGFb, tobacco smoking has long been established to cause lung cancer, and nicotine contained in tobacco smoke is reported to be immunosuppressive. With the discovery of miR183 as a critical NK cell regulator, the goals of the project are to investigate (i) if nicotine associated with tobacco smoking and lung cancer induces the miR183/DAP12 circuit to suppress NK function, (ii) if use of tobacco or e-cigarettes induce miR183 an disrupt NK function, and (iii) if miR183 blockade can be targeted to treat early stage lung cancer by reactivating NK cell function.

What was accomplished under these goals?

Aim 1: To examine the role of miR183 in nicotine inhibition of human NK cell function.
We have earlier uncovered a seminal transforming growth factor –beta (TGFb)/miR-183/DAP12 pathway utilized by tumor cells to silence human NK cells by down-regulating cytotoxicity markers like NKp44 and KIR2DS4. Given that tobacco smoking can suppress immunity, we proposed that nicotine could disrupt human NK cells via a similar molecular pathway. Our initial data provided in the proposal showed promising results where Nicotine and the nicotine metabolite, 4-(methyl-nitrosamine)-1(3-pyridyl)-1 butanone (NNK), reduced intracellular DAP12, surface NKp44, and lysis of A549 lung tumor cells by NK cells.

However, due to Nicotine instability, we have been unsuccessful to repeat the results. We have tested different new lots of pure Nicotine from Sigma, with different donors, without seeing DAP12 and activating markers being reduced. NNK and Nicotine used in e-cigarettes (e-Nicotine) were also tested. By flow cytometry, we found that surface marker NKp44 and KIR2DS4 were not affected by Nicotine or e-Nicotine, although TGFb, used as a positive control, was able to suppress both NKp44 and KIR2DS4 surface expression (Fig. 1A,B) Intracellular DAP12 levels, accessed by western blot and qPCR, remained unchanged despite various treatments with Nicotine and e-Nicotine, and a representative experiment is shown in Fig.1C.

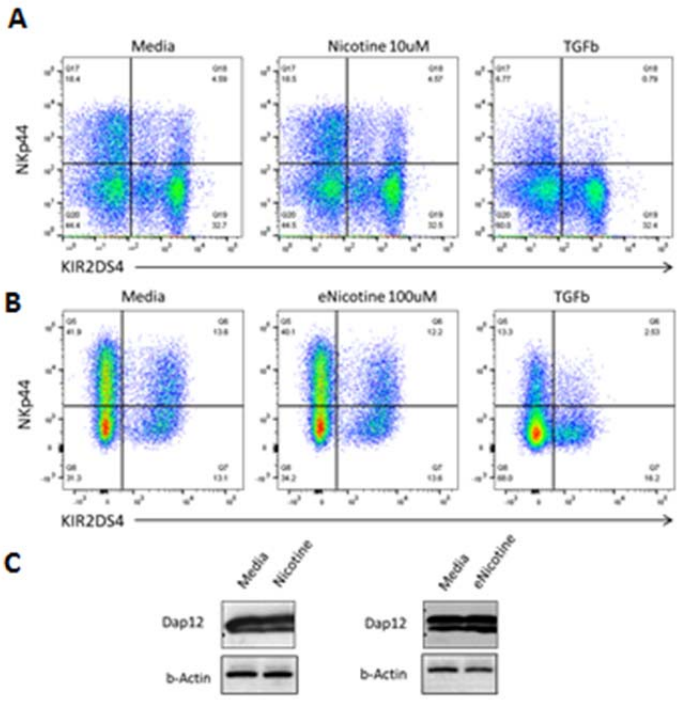

Figure 1. Effect of nicotine and e-nicotine on NK cells. NK cells , treated or untreated with nicotine (A) or e-nicotine (B) for 3 days were accessed by flow cytometry for surface expression of NKp44 and KIR2DS4. TGFb treatment was included as a positive control. (C) Western blot analysis of intracellular DAP12 was also conducted.

Cytotoxic function of NK cells was analyzed by Cr^{51} release assay and showed that NK cells retained their function after being treated with nicotine products. The concentration of Nicotine tested ranged from 1-160 μM, NNK ranged from 1-100 μM and e-Nicotine was from 10-1000 μM. The treatment was extended from 24h to 5 days. Yet, all these conditions did not result in suppression of NK cells, although TGFb treatment, included as a positive control, always effectively inhibited NK receptor expression and tumoricidal function. A representative experiment shows the inability of nicotine to block lytic function in freshly-isolated NK cells and NK92 cell line against A549 human lung tumor cells (Fig. 2A) while another representative experiment depicts similar results of ineffectiveness of e-nicotine in suppressing NK lysis against K562, PCSC, Panc-1 and Mia-Paca tumor cells (Fig. 2B).

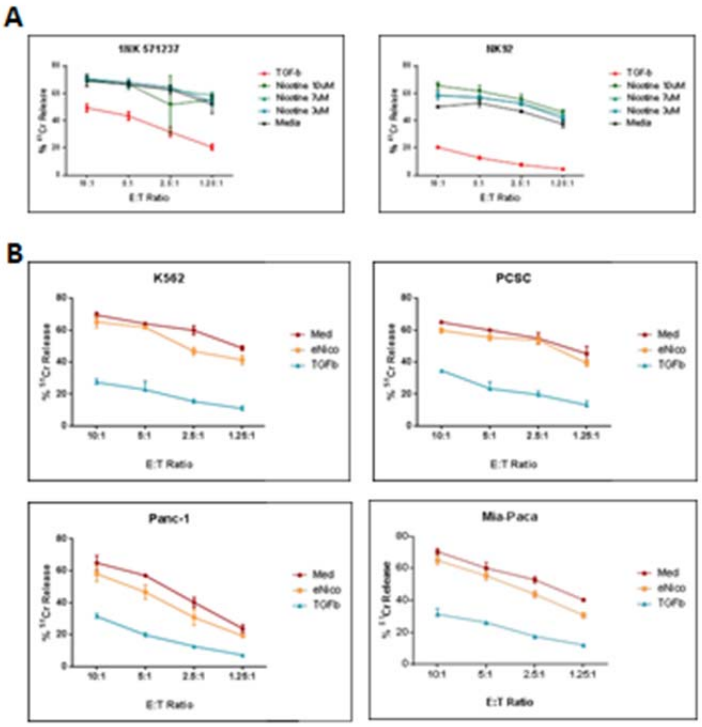

Figure 2. NK cytotoxic function was accessed by a 5 h ^{51}Cr release assay against different cancer cell lines. Primary NK cells and NK92 cells were treated 3 days with A) Nicotine at different concentrations, or B) 10uM eNicotine before being tested for lytic function of primary NK cells against K562, or three pancreatic cell lines, PSCS, Panc-1 or Mia-Paca.

Despite initial experiments suggesting the presence of nicotine receptors on activated human NK cells, we found that treatment of NK cells with Nicotine and NNK with or without nicotine receptors blockade by bungarotoxin (competitively binds to α7/α9 nicotine receptors) and anti-α7/β4 antibody (block α7/β4 nicotine receptors) did not alter the level of NK cells activating markers. Concentration of bungarotoxin and antibodies used ranged from 5-30 μM.

<u>Aim 2. To verify that use of tobacco or e-cigarettes can induce miR183 and disrupt NK function.</u>
Upon approval from DOD Human Research Protection Office of our IRB to conduct the effect of smoking on NK phenotype and function, we began to collect blood samples and have so far processed peripheral blood mononuclear cells from 7 current smokers, 6 e-cigarette users and 3 past smokers. We have frozen all the materials in aliquots with the aim to test all samples together in one experiment for each functional parameter, in order to minimize day-to-day technical variability.

<u>Aim 3. To construct stable anti-sense miR and nanoparticles targeted to human NK cells</u>.
This aim intends to restore NK function against tumor cells by targeting miR183 that is induced in NK cells by TGFb in the tumor microenvironment. In order to answer if nanoparticle-based anti-miR183 can be an effective therapeutic strategy, an in vivo xenograft model must first be optimized to accept and maintain human NK cells We have succeeded in developing better techniques to detect human NK cells in NSG mice by a small but critical change in our protocol. We find that IL15 can markedly enhance human NK cell survival in vivo in NSG mice bearing human A549 lung tumors. Addition of IL15 for the last 24 h of in vitro culture followed by intravenous injection of these NK cells in IL15-containing phosphate-buffered saline into mice altered their ability to survive in the xenogeneic environment. In previous experiments with IL2 alone for in vitro culture, we could barely detect 1-4% human NK cells in the spleen, liver, blood and less than 1% in the tumor site of mice, even though the starting population of 1 x 10^7 NK cells that were 73.8% CD56+NKp46+ and 52.3% NKp44+NKp46+ in the in vitro culture was injected into each mouse (Fig. 3). Addition of IL15 in vitro resulted in higher levels of NK cells, with 94.2% CD56+NKp46+ and 43.6% NKp44+NKP46+. Adoptive transfer of these IL15-activated NK cells resulted in 22.1% human CD56+NKp46 NK cells in blood, 22.1% in liver, 8.47% in spleen but only 0.6% in the tumor bed (Fig. 4). Thus, the human NK cells can mobilize to blood, live and spleen but cannot appear to enter the tumor bed. Using NKp44 as a marker instead of CD56, we detected similar results. We are in the process of evaluating various means

to digest the xenografted tumor to determine if lack of detection of intratumoral NK cells is due to poor recovery rather than true inability to penetrate the tumor bed.

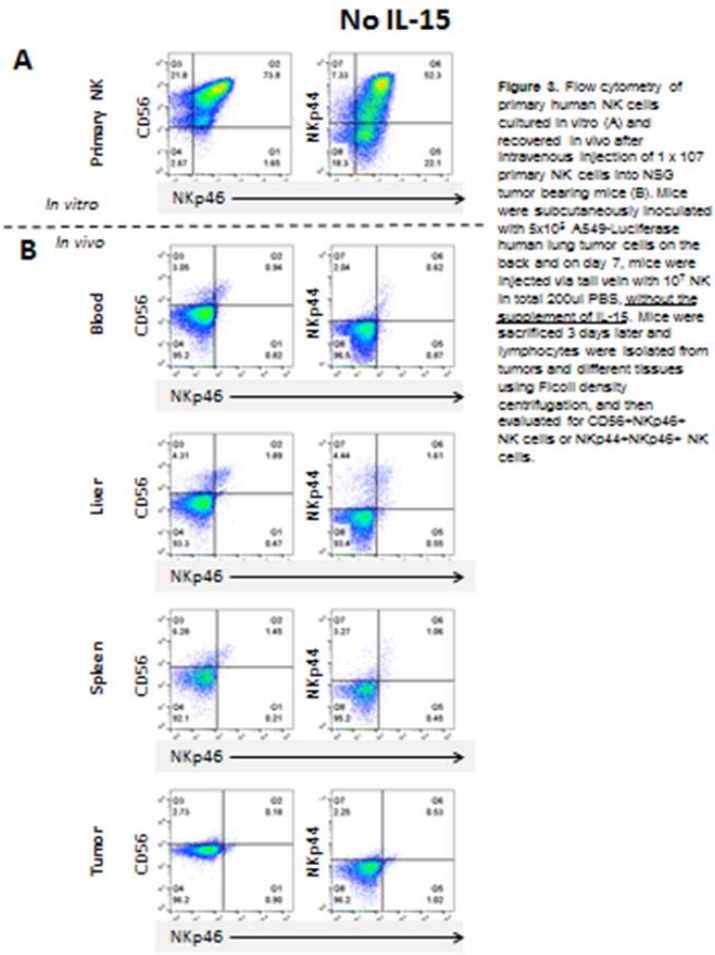

Figure 3. Flow cytometry of primary human NK cells cultured in vitro (A) and recovered in vivo after intravenous injection of 1 x 107 primary NK cells into NSG tumor bearing mice (B). Mice were subcutaneously inoculated with $5x10^5$ A549-Luciferase human lung tumor cells on the back and on day 7, mice were injected via tail vein with 10^7 NK in total 200ul PBS, without the supplement of IL-15. Mice were sacrificed 3 days later and lymphocytes were isolated from tumors and different tissues using Ficoll density centrifugation, and then evaluated for CD56+NKp46+ NK cells or NKp44+NKp46+ NK cells.

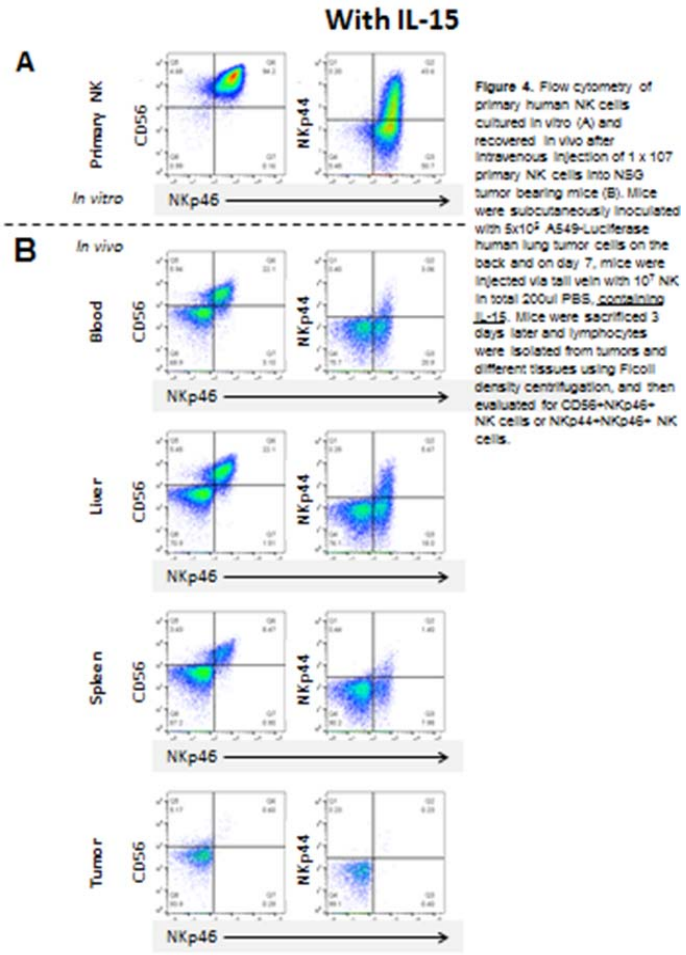

Figure 4. Flow cytometry of primary human NK cells cultured in vitro (A) and recovered in vivo after intravenous injection of 1 x 107 primary NK cells into NSG tumor bearing mice (B). Mice were subcutaneously inoculated with $5x10^5$ A549-Luciferase human lung tumor cells on the back and on day 7, mice were injected via tail vein with 10^7 NK in total 200ul PBS, containing IL-15. Mice were sacrificed 3 days later and lymphocytes were isolated from tumors and different tissues using Ficoll density centrifugation, and then evaluated for CD56+NKp46+ NK cells or NKp44+NKp46+ NK cells.

In terms of nanoparticle-based immunotherapy of lung cancer, the first step has been to determine if PLGA nanoparticles are compatible to NK cells. What is exciting is that PLGA nanoparticles (NP) appear to be easily taken up by human NK cells in vitro and they are not targeted into lysosomes, thus indicating that the anti-sense miR183 cargo within PLGA NPs will not be degraded inside the NK cell. Cytotoxicity studies confirmed that the NPs do not compromise the viability of the NK cells up to concentrations of 1000 ug/mL, which is a much higher dose than what is typically administered.

What opportunities for training and professional development has the project provided?
Nothing to report

How were the results disseminated to communities of interest? Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

Aim 1: To examine the role of miR183 in nicotine inhibition of human NK cell function.

We found that pure Nicotine that is currently available commercially from Sigma is very unstable and had no dramatic effects on NK cell, unlike TGFb, although this product had been effective in the past. However, the pathway that Nicotine and smoking affect/suppress the immune systems remains open to explore and is still critical for us to understand the mechanism of smoking to contribute to lung cancer. We are looking for other sources of nicotine and are consulting colleagues who also are facing the same issues of nicotine instability to see if they have come up with solutions to stabilize the compound. We will not face this issue in Aim 2, where we expect that we will detect some effects of nicotine on NK function in vivo in smokers and have proceeded to conduct the clinical protocol in Aim 2.

Aim 2. To verify that use of tobacco or e-cigarettes can induce miR183 and disrupt NK function.
We will finish collecting the 20 samples per group from never smokers, heavy tobacco smokers, past smokers and e-cigarette users and then test for intracellular DAP12 and surface NKp44 by flow cytometry and for tumoricidal function by Cr-release assay, followed by data analysis.

Aim 3. To construct stable anti-sense miR and nanoparticles targeted to human NK cells.
We are now focusing on loading the PLGA nanoparticles with anti-sense miR183 and obtaining non-viral transfection of the NK cells in vitro, with the aim to achieve optimal nanoparticle formulation for delivery of anti-miR183 in NK cells. Once optimized, we will then employ the anti-miR183-encapsulated nanoparticles for immunotherapy of lung cancer in NSG mice. We are in the process of preparing nanoparticles. We will first preload the nanoparticles into NK cells prior to their intravenous administration into NSG mice bearing A549-human lung tumors. If successful, we will then introduce the nanoparticles in free form given intravenously into tumor bearers that have already been administered NK cells.

4. **IMPACT:**

TGFb has long been known to be immunosuppressive but clinical trials with anti-TGFb or TGFb inhibitor in cancer patients have not produced notable successes, likely due to numerous off-target effects related to the pleiotrophic nature of TGFb with various physiological effects. Direct targeting of miR183 could avoid such issues to constitute a new molecular and precise strategy for lung cancer therapy. Most importantly, we have discovered a new immune checkpoint inhibitor, miR183, that disrupts NK function against cancer.

What was the impact on the development of the principal discipline(s) of the project?

To date, no attempts have been made to address microRNA therapeutics to correct tumor immunity against cancer and our project is of high impact in introducing precision medicine to control a new immune checkpoint inhibitor.

What was the impact on other disciplines? *Nothing to report*

What was the impact on technology transfer? *Nothing to report*

What was the impact on society beyond science and technology? Nothing to report

5. **CHANGES/PROBLEMS:** *Nothing to report*

Changes in approach and reasons for change: Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents. *No changes*

Significant changes in use or care of human subjects. *No changes*

Significant changes in use or care of vertebrate animals. *No changes*

Significant changes in use of biohazards and/or select agents. *No changes*

6. **PRODUCTS:** *Nothing to report*

Website(s) or other Internet site(s): *Nothing to report*

Technologies or techniques: *Nothing to report*

Inventions, patent applications, and/or licenses: *Nothing to report*

Other Products: *Nothing to report*

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

Name:	*Julie Y. Djeu*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*No change*
Funding Support:	*No change*

Name:	*David Drobes*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*No change*
Funding Support:	*No change*

Name:	*Junmin Zhou*
Project Role:	*Research Scientist*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*8*
Contribution to Project:	*No change*
Funding Support:	*No change*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Julie Djeu:
NEW Djeu (PI) 03/19/2016 – 02/28/2019
State of Florida James & Esther King Discovery Science Award $511,569
2.4 Cal. Months
Nanoparticle-based Targeting of miR183 for Immunotherapy of Lung Cancer
Specific Aim 1: Develop human lung xenograft immunotherapy model in NSG mice
We will utilize generic and doxycycline-inducible lentiviral constructs to validate miR-183 targeting of DAP12 in vivo in human NK cells engrafted in NSG mice.
Specific Aim 2: Engineer nanoparticles for delivery of anti-miR-183 into human NK cells
We will utilize poly (lactide-co-glycolide) (PLGA) nanoparticles for 10 encapsulation of stabilized anti-sense miE-183. 2) functionalization with cell penetrants and NK-specific targeting molecules 3) evaluation of functionalized nanoparticles in vitro in NK cells and mixed NK-tumor cell hydrogels as well as in vivo.
Specific Aim 3: Examine therapeutic efficacy of anti-miR-183 encapsulated nanoparticles in NSG mice

The optimized nanoparticles will be tested in a therapeutic setting in NSG mice bearing human A549 tumors, with analysis of miR-183 and DAP12 in human NK cells harvested from mice to verify biological targeting.
Role: PI

COMPLETED
(Djeu/Drobes/Brandon) 05/01/2015 – 01/29/2016
Moffitt Team Science Award
The Role of MicroRNA-183 in Natural Killer Cell Function Against Human Lung Cancer
The goal of this application is to unravel the mechanisms that govern innate immune escape in lung cancer, including differences in immune function between smokers, e-cigarette users, and non-smokers and as a function of smoking cessation.
Role: Co-PI

Kumar (PI) 07/01/2013 – 06/30/2016
Gateway for Cancer Research Foundation $106,244
0.24 Cal. Months
A Phase II Clinical Trial to Ameliorate Symptoms of Cognitive Impairment in Women Treated With Chemotherapy for Breast Cancer: The COGNUTRIN Trial
The current pilot study will be the first clinical trial to examine the safety and effectiveness of a 3-month intervention with a combination botanical and biologic supplement (COGNUTRIN) on cognitive function. This funding from Gateway will enable us to focus directly on the cancer patients' clinical problems and deliver clinical treatments that may prove successful to reverse chemobrain, which is truly relevant issue to cancer patients who are surviving cancer today.
Role: Co-Investigator

David Drobes:
NEW
6JK02 (Drobes) 3/19/16 – 2/28/21
Florida Biomedical Research Program
Facilitating Smoking Cessation with Reduced Nicotine Cigarettes
The goal of this project is to develop and then test a targeted smoking cessation intervention that utilizes pre-quit smoking of reduced nicotine cigarettes.

COMPLETED
(Djeu/Drobes/Brandon) 5/1/15 – 1/29/16
Moffitt Team Science Award
The Role of MicroRNA-183 in Natural Killer Cell Function Against Human Lung Cancer
The goal of this application is to unravel the mechanisms that govern innate immune escape in lung cancer, including differences in immune function between smokers, e-cigarette users, and non-smokers and as a function of smoking cessation.
Role: Co-PI

What other organizations were involved as partners? *Nothing to report*

8. **SPECIAL REPORTING REQUIREMENTS** *Nothing to report*

9. **APPENDICES:** *Nothing to report*